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Implications for Enhanced Training, Operational Performance and Sex-Specific  
Responses

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**14. ABSTRACT**

The specific aim of the second segment of this project series was to determine the impacts of environmental conditions on specific markers of exercise training response and/or acclimation. Untrained males (n=36) served as study participants and were stratified across three training environments 1) control (~20°C, 30% RH), 2) heat (~33°C, 30% RH), or 3) cold (~7°C, 30% RH) environments. Daily training was prescribed for a period of 14 days. Following a controlled diet and exercise plan the day before and after overnight fast participants reported to the laboratory in the early morning hours. A muscle biopsy was obtained from the vastus lateralis (randomized, counter-balanced leg) before starting exercise (Pre) – (days 1 and 15). Participants then exercised for 60 minutes at 60% of maximal aerobic capacity in one of the above-described environments. At the conclusion of the exercise trial, participants recovered (seated rest, under normal laboratory conditions) for four hours before a final muscle biopsy was taken (days 1 and 15). The additional four hours was necessary for the genes of interest to peak following exercise as previously described. Data was analyzed using a mixed design ANOVA with repeated measures between the training groups and across time, two-way 2 x 3 ANOVA (time x trial). A probability of type I error of less than 5 % was considered significant ( $p < 0.05$ ). At the time of this report, only a portion of the data has been analyzed. However, we have completed all study participant training and testing for this phase of the study (males).

**15. SUBJECT TERMS**

training adaptations, muscle gene response, oxidative stress, environmental stress.

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## **Evaluation of the physiological challenges in extreme environments: Implications for enhanced training, operational performance and sex-specific responses**

### **1. INTRODUCTION:**

In addition to high altitude, cold stress and mountain warfare, heat stress and desert warfare are persistent realities as the U.S. continues its war on terror. However, the physiological responses/adaptations to environmental heat and cold stress during training at the level of the human skeletal muscle are relatively unknown. Our previously funded DOD work has described the acute response of exercise in hot, cold, and neutral temperature environments. In summary of this research we have observed a temperature dependent response with a more favorable response in the colder environments and a blunted response in hot environments. This initial proof-of-concept research is limited by evaluation of only the acute response to exercise. In order for successful implementation into military training and operations the chronic response(s) must be evaluated. Furthermore, actual performance based measures must be evaluated after a period of training/acclimation.

The aim of this project is to translate our previous temperature dependent response to a short term (14 day) training protocol to determine if the observed acute muscle and oxidative stress responses translate to enhanced training adaptation and/or increased physical performance. These results may suggest a temperature optimized training response and/or a temperature mediated delay in training adaptations. This study moves the focus of our research (and others) from a descriptive approach to an approach that aims to mitigate the known consequences of performance in different temperatures to novel approaches to mitigate these effects thereby providing field ready solutions.

### **2. KEY WORDS:**

Skeletal muscle gene response, mitochondria, oxidative stress, heat stress, cold stress, exercise

### **3. ACCOMPLISHMENTS:**

We have remained on schedule for the majority of our study participant testing. There have only been slight delays in our sample analyses procedures due to the relocation of Dr. John Quindry from Auburn University to the University of Montana. Year 2 was also complicated by the dual location testing procedures to accommodate the desired sample size for the environmental training study methodologies. Below represents an up to date sequence of accomplishments progressing through the completion of data collection and the initial analyses for the first years project.

1. The University of Montana approved the IRB for study 2, "Effects of Environmental Temperature on Exercise Response and Adaptation," on 9

December 2016 and all of the supplies and necessary testing equipment was set up, tested and calibrated in preparation for the upcoming data collection.

2. The Army HRPO approval for the study was received on 28 December 2016.
3. Data collection for study 2 was initiated in early February and included an initial n=6 study participants. The protocol including initial testing, 14 days of training (environment specific) and post training testing was completed on March 13.
4. Data collection for study continued starting with our next round of study participants (n=6) on May 27 and was completed on May 3.
5. The University of Nebraska Omaha retained a similar schedule for all initial testing.
6. Sample analyses for the first round of testing has been completed and the remainder of the samples will be completed throughout the remainder of the academic year (spring 2018).
7. The second round to testing was initiated in the late summer (UNO) and early fall semester (UM) and has recently been completed (November, 2017).
8. A progress report was delivered at Fort Detrick in October of 2017 with Ruby and Slivka attending.
9. All testing was completed by the end of November 2017 for study 2.
10. Sample analyses for study 2 (muscle and blood samples) are ongoing and is expected to be completed by the end of the 2018 spring semester.
11. One abstract has been prepared and submitted to the National ACSM meeting for June of 2018 presentation. An initial manuscript from phase 1 of the study series has been prepared. Both are presented in the products section of this document.

## Methodology:

The following represents the basic methodology for the data collection surrounding phase 2 of the study series.

### **Study 2: Effects of environmental temperature on exercise response and adaptation.**

We have previously determined that the skeletal muscle response to exercise varies depending on the ambient conditions (hot, neutral, cold) and the temperature of the muscle. For example PGC1 $\alpha$  was increased to a greater degree in the cold compared to the neutral environment. In contrast, exercise in a hot environment diminished the PGC1 $\alpha$  response. These data suggest that the adaptive potential of the skeletal muscle may be advanced and/or decreased depending on the training environment. To more effectively address this question, active males were exposed to a short-term training period under varied environmental conditions.

*Participants.* Participants include 36 untrained males. Testing occurred at two locations (Montana and Nebraska) using identical equipment and protocols in order to accumulate the number of subjects needed in a time efficient manner. All participants provided written informed consent approved by the University Institutional Review Board and USAMRMCC Office of Research Protections prior to commencement of testing.

*Experimental Design.* Subjects were matched across three training conditions 1) control (~20°C, 30% RH), 2) heat (~33°C, 30% RH), or 3) cold (~7°C, 30% RH) environments. Daily training was prescribed for a period of 14 days.

*Protocol.* Following a controlled diet and exercise plan the day before and after overnight fast participants reported to the laboratory in the early morning hours. A muscle biopsy was obtained from the vastus lateralis (randomized, counter-balanced leg) before starting exercise (Pre) – (days 1 and 15). Participants then exercised (cycle ergometer) for 60 minutes at 60% of maximal aerobic capacity in one of the above-described environments. At the conclusion of the exercise trial, participants recovered (seated rest, normal laboratory conditions) for four hours before a final muscle biopsy was taken (days 1 and 15). Daily training (60 minutes of self-selected intensity) proceeded with days off for Saturday and Sunday for a total of 14 training sessions. Data were analyzed using a mixed design ANOVA with repeated measures between the training groups and across time, two-way 2 x 3 ANOVA (time x trial). A probability of type I error of less than 5 % will be considered significant ( $p < 0.05$ ).

### *Participants*

36 untrained males were recruited from the university and local communities to take part in the study. Participants were required to pass a pre-screening Physical Activity Readiness-Questionnaire. Participants signed an informed consent form that was approved by the university Institutional Review Board and the Army HRPO office.

## ***Preliminary Testing***

### ***Hydrodensitometry***

Body composition was assessed via an underwater weighing tank (Exertech, Dresbach, MN) utilizing estimated residual volume based on height and weight. Participants were required to fast for  $\geq 3$  hours prior to testing. Dry weight was determined using a digital scale (Befour Inc., Cedarburg, WI) and height was measured. Participants were weighed while completely submerged. Body density and percent body fat were estimated using the Siri equation.<sup>17</sup>

### ***Peak Aerobic Capacity***

Participants arrived at the lab fasted for  $\geq 3$  hours prior to  $\text{VO}_2$  peak testing. A cycling graded exercise test, was performed on a treadmill ergometer (TMX225C, Fullvision, Inc., Newton, KS)<sup>18</sup> while participants' expired gas was analyzed every 15 seconds by a metabolic cart (Parvomedics, Inc., Sandy, UT). Heart rate was monitored and recorded using a heart rate watch and chest strap (Polar Electro, Kempele, FL).

## ***Experimental Protocol***

Following the completion of the initial baseline testing, subjects were stratified into one of three training environments. Training occurred over three semesters with hot and control training occurring during colder winter months and cold training occurring during late spring and early fall. Testing on days 1 and 15 proceeded at the assigned environmental conditions 1) control ( $\sim 20^\circ\text{C}$ , 30% RH), 2) heat ( $\sim 33^\circ\text{C}$ , 30% RH), or 3) cold ( $\sim 7^\circ\text{C}$ , 30% RH) environments. Upon arrival to the laboratory an initial muscle biopsy (vastus lateralis) was obtained along with a pre-exercise blood sample. Study participants were then equipped with temperature sensors (skin, chest location; core, rectal) and a chest strap heart rate monitor. Pre and post exercise measures of nude body weight were also obtained.

Exercise proceeded in the prescribed environment using a Tesco environmental chamber at each testing location for a period of 60 minutes (60% of peak  $\text{VO}_2$ ). Immediately following the exercise session, study participants exited the environmental chamber and a post-exercise muscle biopsy and blood sample were obtained. Following sample collection, the temperature sensors were removed and a post-exercise measure of nude body weight was obtained. Exercise recovery proceeded under normal laboratory conditions for a period of four hours with a subsequent blood sample obtained 1-hour post exercise. At four hours post-exercise, a final blood and muscle sample were obtained.

All initial testing (pre-training) occurred on a Monday with the following 14-week days serving as training days. Training was scheduled for 60 minutes each session in the same environment as the prescribed testing conditions (T-F for week 1, M-F for weeks 2 and 3). Post-training testing was completed on the following Monday and included the identical procedures indicated above at the same absolute workload.

No less than two days after completing the post-training experimental trail, study participants returned to the laboratory for subsequent measures of body composition and peak  $\text{VO}_2$  using the identical procedures described above.



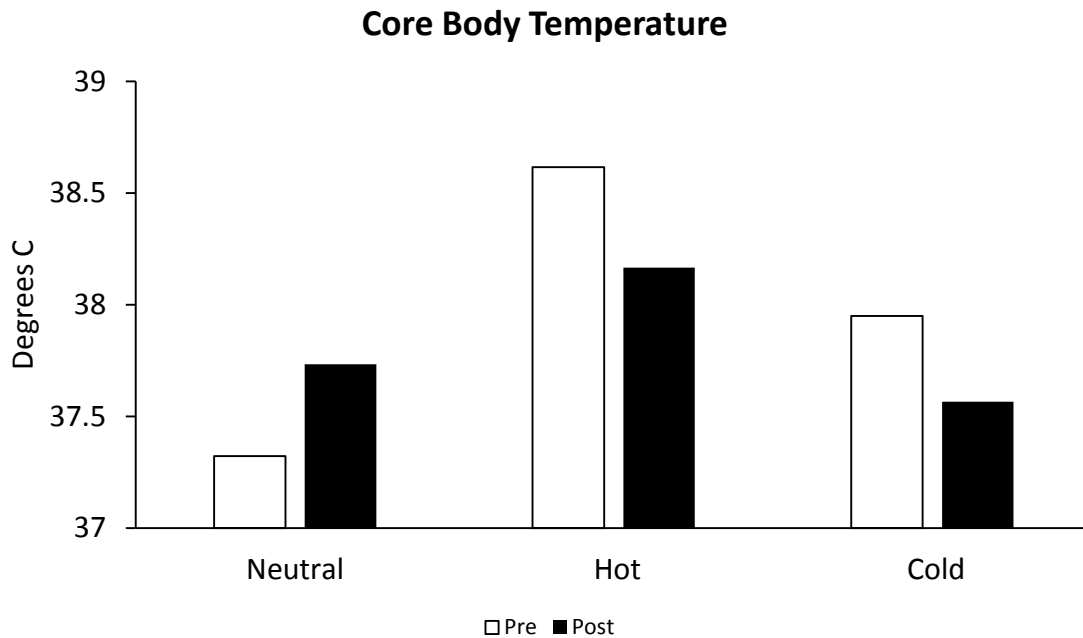
## Results:

The results at the time of this report are not fully complete. The descriptive data from the study participants are reported in Table 1.

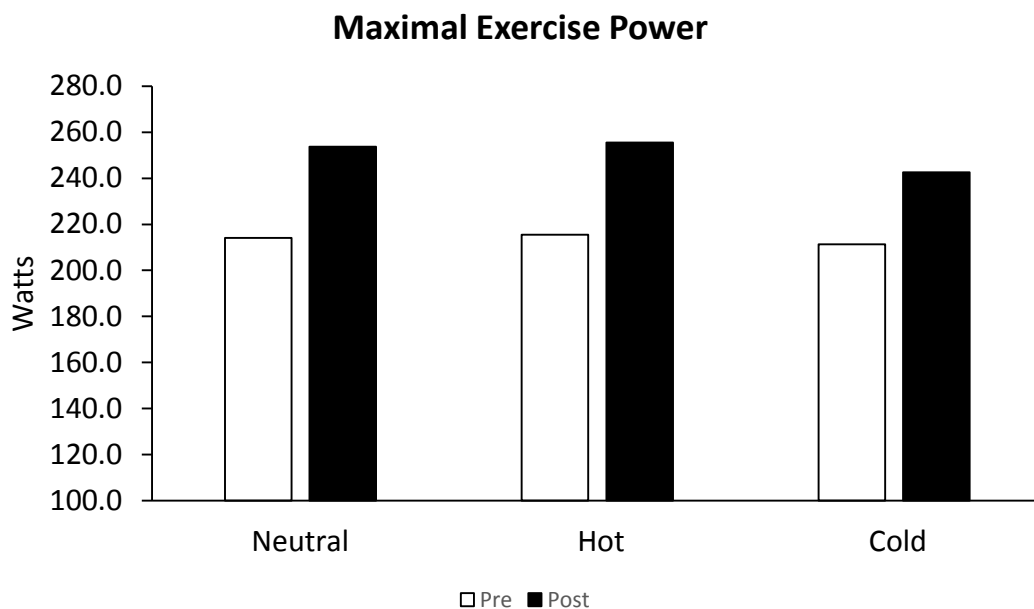
**Table 1.** Descriptive data from study participants.

	Pre	Post	Change
<b>Neutral n=12</b>			
Age (y)	23 ± 4		
Height (cm)	176 ± 7		
Weight (kg)	85 ± 26	86 ± 27	No Change
<b>Hot n=12</b>			
Age (y)	25±5		
Height (cm)	178 ± 6		
Weight (kg)	86 ± 19	86 ± 18	No Change

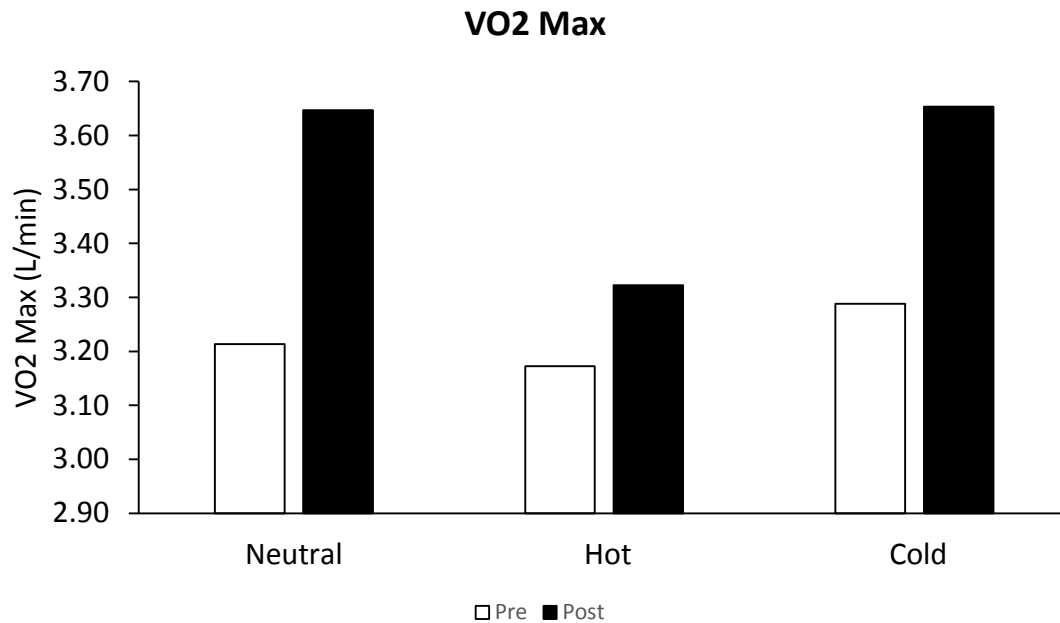
\* Current data not yet analyzed for the Cold training group.



**Figure 1.** Measures of peak core body temperature during the experimental trials. (n=12, 12 for neutral and hot, respectively; n=6 for cold).

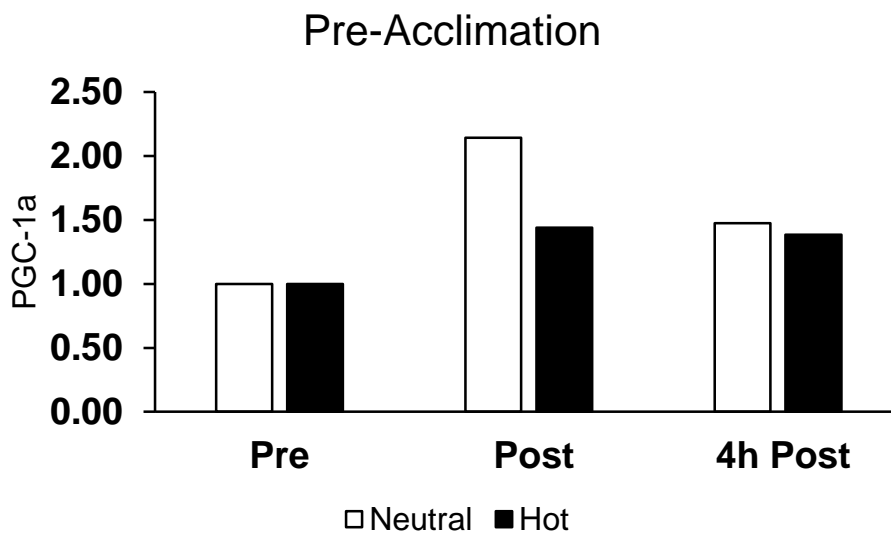


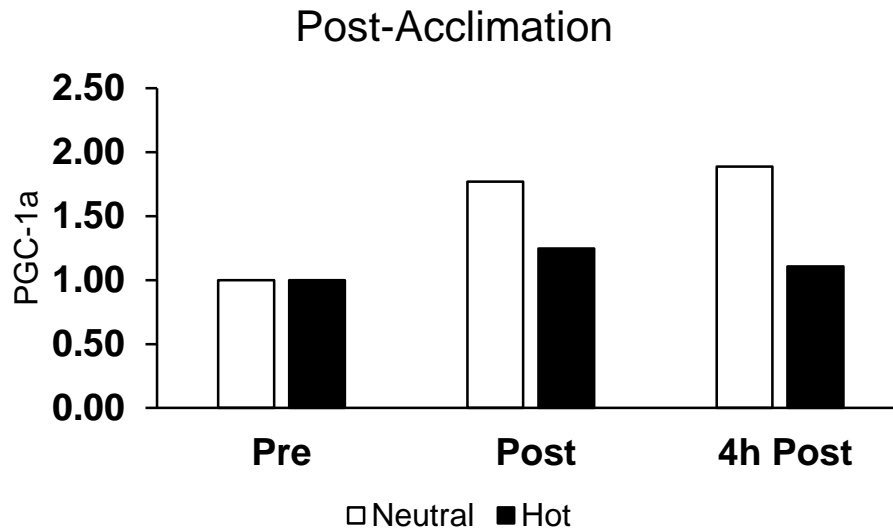
**Figure 2.** Measures of peak power during the pre and post-training graded exercise trial. Each training group increased peak power output by approximately 40 watts. (n=12, 12 for neutral and hot, respectively; n=6 for cold).



**Figure 3.** Measures of VO2 peak during the pre and post-training graded exercise trial. (n=12, 12 for neutral and hot, respectively; n=6 for cold).

Only a small portion of the total samples have been analyzed at the time of this report. The remaining samples (muscle and blood) are currently being analyzed.





**Figure 4.** Changes in PGC-1α pre and post environmental acclimation. (n=12 for neutral and hot, respectively; n=6 for cold).

Although these data only demonstrate partial sample analyses for the control (neutral) and hot training groups, these preliminary data demonstrate a partial inhibition of the PGC-1α pathway and may indicate a theoretical reduction in training responsiveness. These data may indicate that training in the heat may decrease the potential for peripheral derived training responses compared to the control environment. Once finalized (when all cold training group samples have been analyzed), these data may indicate environmental specific central and peripheral responses.

## **Opportunities for Training and Professional Development:**

Nothing to Report

## **Dissemination of results:**

The initial dissemination from phase I of the study series (oxidative stress) has been prepared and submitted for presentation the National American College of Sports Medicine meeting in June 2018. The abstract prepared for submission are provided in the products section of this report.

Additionally, an initial draft of the first manuscript has been prepared for submission and also attached in the products section of this report.

## **Plans for the next reporting period:**

The next reporting period will include near final sample analyses for phase 2 of this study series. We are also preparing university IRB revisions for inclusion of 24 additional study participants to continue the environmental training response study. Once university IRB approvals are obtained, these revised protocols will be prepared for ARMY HRPO review and approval. It is anticipated that testing will commence for this second phase of training in February 2018.

## **4. IMPACT:**

The impact of this initial project is yet to be determined based on the early, initial results. However, these data will provide foundational research describing the impacts specific exercise approaches may act to counter the deleterious impacts of high altitude. Moreover, at present, minimal sex specific responses have been noted. This may impact programmatic procedures related to training methodologies during high altitude deployments and be uniformly applied to male and female warfighters.

### **Impact on the development of the principal discipline**

Nothing to report at this time.

### **Impact on other disciplines**

Nothing to report at this time.

### **Impact on technology transfer**

Nothing to report at this time.

## **Impact on society beyond science and technology**

Nothing to report at this time.

## **5. CHANGES/PROBLEMS:**

There have been limited changes and problems associated with our year 2 effort. Despite the rigorous training schedule, compliance of the study participants at both locations was excellent. Any testing sessions that were missed due to schedule conflicts were easily rescheduled during the weekends throughout the 14 days of training.

The only change to the upcoming SOW is the continuation of this aggressive environmental stress study and the inclusion of female study participants. Two groups of female study participants will be evaluated during heat and control environments as indicated above. This will increase our overall sample size to an  $n=24$  in each of the hot and control environments ( $n=12$  males, 12 females in both).

This requested change to the original SOW was discussed at the annual program review in October 2017 and also with our program officer. Despite this change in SOW, the total number of samples for analyses and the accompanying expected workload will be slightly increased. However, we do not anticipate any budget alterations at this time. Moreover, this change in the SOW will increase the overall ability to consider environmental periodization and/or sex specific responses to training adaptations across varied environments.

### **Changes in approach**

No changes were made for year 2 of the project.

### **Delays and resolutions**

The only delays associated with year 2 activities are associated with the re-location of one of our co-investigators (John Quindry). Dr. Quindry left Auburn University and accepted a position here at the University of Montana. Due to the necessary transition and lab set up, Dr. Quindry had been slightly delayed in his analyses of the samples for markers of oxidative stress.

### **Changes on expenditures**

Nothing to report

### **Changes in human subjects**

No changes in human subjects occurred for year 2 of the project.

## 6. PRODUCTS:

At this point, one abstract has been submitted to ACSM for presentation at the annual meeting June 2018.

### **Blood Oxidative Stress Following Exercise Recovery in Normobaric and Hypobaric Hypoxic Environments**

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**Purpose:** Hypobaria and hypoxia exert independent effects on exercise-induced oxidative stress in blood, while the hypobaric and hypoxic influences are not well defined. The current study was undertaken to quantify exercise-induced oxidative stress recovery during lab-simulated hypoxic and hypobaric conditions following a common bout of exercise. **Methods:** At a base elevation of 975m, physically active participants (n=16), ages 18-40, provided informed consent prior to performing 60 minutes of cycle ergometry at 70% watts max. Using a randomized counter-balanced crossover design participants recovered for 4 hours in 3 lab-simulated conditions; 1000m normobaric normoxia (NN, 675mmHg, 18.8%FiO<sub>2</sub>), 4400m normobaric hypoxia (NH, 675mmHg, 12% FiO<sub>2</sub>), or 4400m hypobaric hypoxia (HH, 440mmHg, 12% FiO<sub>2</sub>). O<sub>2</sub> saturation was confirmed via pulse oximetry throughout the 3 exercise-recovery trials. Blood samples were collected in heparinized vacutainer tubes at time points Pre, Post, 2 Hours Post, and 4 Hours Post exercise. Blood plasma was analyzed for the quantification of oxidative stress to proteins (protein carbonyls, PC; 3-nitrotyrosines, 3NT), lipid (lipid hydroperoxides, LOOH; 8-isoprostanes, 8-ISO), and antioxidant capacity (ferric reducing ability of plasma, FRAP; trolox equivalent antioxidant capacity, TEAC). **Results:** Plasma TEAC, FRAP, 3NT and PC were unaltered by exercise and recovery environments (p>0.05). Exercise-induced increases in LOOH and 8-ISO were observed, although time-by-trial differences were not present. **Conclusion:** These data indicate that exercise recovery in simulated conditions of NH and HH do not impact a common panel of blood oxidative stress measures.

Funding provided by the Department of Defense United States Army Medical Research and Materiel Command (DOD USAMRMC: W81XWH-15-2-0075).

**Skeletal Muscle mRNA Response to Hypobaric and Normobaric Hypoxia After  
Exercise**

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### **Abstract**

**Aim:** To determine the effects of recovery in hypobaric hypoxia (HH), normobaric hypoxia (NH), and normobaric normoxia (NN) after exercise on gene expression related to mitochondrial biogenesis, myogenesis, and proteolysis. **Results:** SpO<sub>2</sub> was lower in HH (76.02 ± 0.58%) than NH (79.45 ± 0.56, p<0.001), which were both lower than in NN (96.3 ± 0.17, p<0.001). Heart rate was higher in HH (82 ± 2 bpm) than NH (77 ± 1 bpm, p<0.001), which were both higher than in NN (67 ± 1 bpm, p<0.001). TFAM was unaltered in normobaric normoxia (p=0.465) but increased after HH (p=0.037) and NH (p=0.006) exposure with no differences between HH and NH (p=0.501). MSTN decreased from pre- to post-exercise (p<0.001) in all conditions and was lower in HH compared to NH (p=0.036) and NN (p=0.017). No other differences were noted in genes related to mitochondrial biogenesis, myogenesis, or proteolysis (p>0.05). **Conclusion:** Recovery in HH after exercise appears to have a greater effect on muscle oxygen transport (SpO<sub>2</sub> and heart rate) than NH. Furthermore, MSTN tends to be further attenuated in HH than NH. Caution should be used when translating data obtained in a NH environment to a HH environment.

**Keywords:** Altitude, gene expression, mitochondrial biogenesis, myogenesis, proteolysis, environmental

## **Introduction**

The environmental conditions that recovery takes place in after exercise may have implications on the cellular and physiological outcomes of that exercise bout. Previous research from our lab have investigated a number of skeletal muscle responses following recovery from exercise in environmental temperatures (D. Slivka, Dumke, Tucker, Cuddy, & Ruby, 2012; Zak et al., 2016), local temperature application (T. J. Tucker, Slivka, Cuddy, Hailes, & Ruby, 2012), and normobaric hypoxia environments (D. Slivka et al., 2013; D. R. Slivka et al., 2014). However, achieving a hypoxic environment through a lowered oxygen fraction may be physiologically different from terrestrial high altitude and thus, has questioned the research outcomes in simulated normobaric hypoxia environments. It is unknown if recovery from exercise in hypobaric hypoxia (HH) produces differences in the skeletal muscle mRNA responses compared to normobaric hypoxia (NH) or normobaric normoxia (NN).

Limited research has directly compared these different forms of hypoxia and a clear understanding is still lacking. For example, research investigating acute HH and NH exposure shows no differences in heart rate (Evetts et al., 2005; Self, G Mandella, Prinzo, M Forster, & Shaffstall, 2011), breathing frequency, or arterial oxygen saturation (Evetts et al., 2005). This suggests both are viable methods for simulating a hypoxic response. Alternatively, resting in NH for 10 hours may produce higher ventilation rates than HH (Loeppky et al., 1997). Additionally, 1 week of NH sleep acclimation has shown no

translational benefits to a HH environment; suggesting they cannot be used interchangeably (Fulco et al., 2011). This disconnect in the response to HH and NH establishes a need for further detail on specific physiological pathways that may be differentially affected.

One such pathway is the production of new mitochondria. Mitochondrial biogenesis occurs with cellular stress such as exercise (Irrcher, Adhihetty, Joseph, Ljubicic, & Hood, 2003; Wright et al., 2007). However, hypoxic stress have demonstrated decrements in mitochondrial development (Ferretti, 1990; Hoppeler et al., 1990; Howald et al., 1990; Kayser, Hoppeler, Claassen, & Cerretelli, 1991; D. Slivka et al., 2013). Previous evidence suggests that NH recovery after exercise increases mitochondrial related gene expression to the same extent as NN recovery (D. R. Slivka et al., 2014). However, this study design did not compare these responses to a HH recovery environment. Therefore, it is currently unknown if HH recovery from exercise would regulate mitochondrial mRNA differently from NH or NN recovery.

The differences in muscle mass regulating genes between HH and NH exposure has not been established. Hypoxic conditions lead to specific signaling events with functional significance in skeletal muscle (Hoppeler, Klossner, & Vogt, 2008). Hypoxia activates proteolytic regulator genes such as FOXO3 to initiate cell death (Bakker, Harris, & Mak, 2007; de Theije, Langen, Lamers, Schols, & Kohler, 2013). Yet research exists' that refute hypobaric hypoxia's role in the activation of proteolysis in mice (Favier et al., 2010) and humans (Manimmanakorn et al., 2013). Contradiction similarly exists on hypoxia's role in the regulation of myogenesis. Myogenic gene expression induces myoblast differentiation and ultimately myotube formation under habitual conditions (Kim, Kosek, Petrella, Cross, &

Bamman, 2005). Hypoxia exposure, however, impairs myoblast differentiation and attenuates myofiber development in female rats exposed to HH (Chaillou et al., 2014) and cultured mouse cells under NH (Di Carlo et al., 2004; Yun, Lin, & Giaccia, 2005). Alternate literature suggests hypoxic exercise improves myogenic activity by promoting muscle growth and repair in humans (Manimmanakorn et al., 2013).

Physiological differences in skeletal muscle gene expression between HH compared to NH and NN recovery after exercise are yet understood. Therefore, the purpose of this study is to determine the response of key genes related to mitochondrial biogenesis and muscle mass regulation during HH, NH, and NN recovery after exercise. Determining the cellular responses in both hypoxic forms will enhance the current literature and advance the understanding of hypoxia's effects on mitochondrial development and skeletal muscle mass regulation. These data may have implications in training strategies and the interpretation/integration of research data obtained using differential methods of achieving hypoxia.

## **Methods**

### *Subjects*

Eight recreationally trained male and seven recreationally trained female subjects participated in this study. They were required to be between the ages of 18 and 40 years old and have a  $VO_{2peak}$  of at least 45 ml/kg/min. Those who previously experienced serious acute mountain sickness, or had a known risk factor for coronary artery disease assessed through a physical activity readiness questionnaire (PAR-Q) were excluded from the study. Furthermore, female participants taking birth control influencing hormonal status or those

who had not had a regular menstrual cycle in the past eight months were excluded. Subjects signed the Institutional Review Board (IRB) and USAMRMC Research Protections Office approved informed consent form prior to testing.

### *Preliminary Testing*

Descriptive data included height (Seca 213 Stadiometer, United Kingdom), weight (Befour PS-660 ST Digital Scale, Saukville, WI), body composition and  $VO_{2peak}$ . Body composition for each subject was assessed through hydrostatic weighing using an electronic load cell-based system (Exertech, Dresbach, MN) correcting for estimated residual lung volume. Body density from this underwater weigh was converted to percent body fat using the Siri equation (Siri, 1993). Peak oxygen uptake ( $VO_{2peak}$ ) was obtained for each participant using a graded exercise protocol starting at 95 watts and increasing by 35 watts every 3-min on an electronically braked Velotron, cycle ergometer (RacerMate, Seattle, WA). Cycling continued until volitional fatigue and the highest obtained oxygen uptake value was considered the  $VO_{2peak}$ . Maximum wattage ( $W_{max}$ ) was calculated by taking the time completed in the last stage divided by the total stage duration (3-min) multiplied by 35 watts and added to the watts of the last completed stage. Expired gases were analyzed every 15-seconds throughout the exercise test using a flow and gas calibrated metabolic cart (Parvomedics TrueOne 2400, Sandy, UT).

### *Experimental Protocol*

All subjects completed three trials in a randomized, counter-balanced order. Each trial was separated by approximately 7 days to allow for biopsy recovery and to minimize carryover acclimation between trials. Subjects reported to the laboratory in the early

morning following an overnight fast. Subjects maintained a 24-hour dietary and 48-hour activity log prior to the first trial and replicated these for the subsequent trials. The exercise trial consisted of a 60-minute bicycle ride on a cycle ergometer at a constant intensity of 70% of their power associated with  $\text{VO}_{2\text{peak}}$ . Each subject drank water ad libitum during the first ride and the amount was replicated for all subsequent trials. Following cessation of exercise, four hours of recovery occurred inside a small (32 in. x 7 ft.) tube shaped altitude chamber (Engineering Innovations, LLC, Littleton, CO) capable of lowering barometric pressure to simulate HH. This tube was located inside of an oxygen-controlled environmental chamber (Tescor, Warminster, PA) capable of lowering the percentage of oxygen in the air to simulate NH. They received one short break after 2 hours of recovery. The experimental recovery conditions were simulated to the following altitudes:

1. NN; 975 m (3,200 ft.) Atmospheric conditions by having both the hypobaric tube and oxygen controlled chamber off so that participants breathed ambient air.
2. HH; 4,420 m (14,500 ft.) hypobaric hypoxia by having the hypobaric tube depressurized altering the barometric pressure while the oxygen-controlled chamber was set to off.
3. NH; 4,420 m (14,500 ft.) normobaric hypoxia by having the hypobaric tube off and the oxygen controlled chamber on.

#### *Oxygenation saturation and Heart Rate*

Tissue oxygen saturation and heart rates were measured using a finger pulse oximeter (Nonin WristOx2 3150, Plymouth, MN) during exercise and again every 60-min during passive recovery. Oxygen saturation and heart rates were recorded on the hour by

having the device placed on the finger and allowed to stabilize for approximately 30 seconds.

### *Biopsies*

Muscle biopsies were taken from the *vastus lateralis* before exercise and after 4-hours of recovery in each trial. The second muscle biopsy was extracted from a separate incision ~2 cm proximal to the pre-exercise biopsy. Each sample was extracted using a 5 mm Bergstrom percutaneous biopsy needle with the aid of suction. The leg was chosen in a random, counter-balanced order. After cleaning the site, approximately 3-4 ml of 1% lidocaine was injected under the skin surface and around the muscle fascia before a small incision through the skin and muscle fascia was made. Once the muscle tissue had been obtained, the sample was quickly cleaned of excessive blood, connective tissue and fat before being placed in All-protect (Qiagen, Hilden, North Rhine-Westphalia, Germany). Samples were placed overnight at 4 °C and then transferred to -30 °C for storage. All subsequent trials repeated this process by alternating between legs.

### *Muscle Sample Preparation and qRT-PCR*

A piece of skeletal muscle ( $11.5 \pm 1.7$  mg) was homogenized in 500  $\mu$ L of Trizol (Invitrogen, Carlsbad, CA) using an electric blender homogenizer (Bullet Blender, Next Advance, Inc, Averill Park, NY) utilizing 1.5 mL Red RINO tubes prefilled with RNase free ceramic beads (Next Advance, Inc, Averill Park, NY). The samples were centrifuged at 12,000 g for 15-min and the aqueous phase then transferred to a fresh 1.5 mL tube and incubated overnight at -20 °C. Samples were centrifuged the next morning and the supernatant was removed. Ethanol was added followed by a 5-minute centrifugation at

7,500 g. The ethanol was then removed, the pellet dried, and then re-dissolved in 30  $\mu$ L of RNase-free water. RNA concentration was quantified using a nano-spectrophotometer (nano-drop ND-2000, Thermo Scientific, Wilmington, DE). Average RNA yields were  $165.9 \pm 12.1$  ng $\cdot\mu$ L<sup>-1</sup>. The average absorbance ratio at 260:280 was  $1.90 \pm 0.00$  indicating high purity of the RNA. The RNA integrity of the samples were assessed using an Agilent RNA 6000 Kit and a 2100 Bioanalyzer (both from Agilent Technologies, Santa Clara, CA) according to manufacturer's instructions. The RNA integrity number (RIN) was  $8.0 \pm 0.1$  indicating the RNA was intact. First-strand cDNA synthesis was achieved using the Superscript IV-first strand synthesis system for RT-PCR kit (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. The resulting cDNA was diluted with the appropriate amount of RNase-free water to achieve a final cDNA concentration of 0.5  $\mu$ g/ $\mu$ L in the PCR reaction. Each 10  $\mu$ L qRT-PCR reaction volume contained 0.5  $\mu$ L of probe and primer mix (PrimeTime qPCR assay Integrated DNA Technologies, Coralville, IA), 5  $\mu$ L qPCR Master Mix (Integrated DNA Technologies, Coralville, IA), and 4.5  $\mu$ L of sample cDNA. PCR was run in triplicate on a Startagene mx3005p PCR system (Agilent Technologies) and a 2-step protocol (1 cycle at 95 °C for 5 s followed by 60 °C for 20 s for 50 cycles). Mitochondrial biogenesis related genes included peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ), estrogen-related receptor alpha (ERR $\alpha$ ), GA-binding protein alpha (GABPA), nuclear respiratory factor 1 (NRF-1), and mitochondrial transcription factor A (TFAM). The myogenic genes of interest involved in muscle hypertrophy are myogenic differentiation factor (MYOD), myostatin (MSTN), myogenin (MYOG), myogenic factor 5 (MYF-5), and myogenic factor 6 (MYF-6). Proteolytic



genes of interest involved in muscle atrophy are forkhead box O3 (FOXO3), atrogin-1, and muscle ring finger 1 (MuRF-1).

Quantification of mRNA for genes of interest were completed using the  $2^{-\Delta\Delta CT}$  method (Livak & Schmittgen, 2001). For each participant, the geometric mean of five housekeeping genes: beta-actin (ACTB), beta-2-microglobulin (B2M), cyclophilin (CYC), ribosomal protein S18 (RPS-18), and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used as the stable reference point. This combination of genes was determined to be stable using NormFinder software (Andersen, Jensen, & Orntoft, 2004). Probe and primer sequences used for qRT-PCR are presented in **Table 1**.

### *Statistical Analysis*

Differences in gene expression between HH, NH, and NN condition trials were analyzed using two-way (time x trial) repeated-measures ANOVAs. In the event of a significant F-ratio, Fisher's protected least significant difference method was applied to determine where differences occurred. All ANOVAs were performed using the Statistical Package for Social Sciences software (SPSS) for Windows Version 23.0 (Chicago, IL). A probability of less than 5% was considered significant ( $p < 0.05$ ). All data are reported as mean  $\pm$  SE.

## **Results**

### *Participant Descriptive Data*

Eight recreationally trained male and seven recreationally trained female participants ( $n = 15$ ) completed this study. Male subjects were taller ( $p < 0.001$ ), weighed more ( $p = 0.028$ ), had a lower percent body fat ( $p = 0.003$ ), a higher peak oxygen uptake ( $p$

= 0.001), and a higher cycling workload at  $VO_{2peak}$  ( $p < 0.001$ ) than female subjects. There were no other differences between males and females ( $p < 0.05$ ). Therefore, male and female subjects were pooled together for analysis. Descriptive data are presented in **Table 2**.

### *Oxygen Saturation and Heart Rate*

No differences in arterial oxygen saturation occurred between trials at baseline, during exercise, or immediately post-exercise ( $p > 0.05$ ) as no experimental interventions had been introduced at these time-points. During each hour of recovery, arterial oxygen saturation in NN was higher than both HH ( $p < 0.001$ ) and NH ( $p < 0.001$ ). Furthermore, oxygen saturation was lower in HH compared to NH at hours one ( $p = 0.004$ ), three ( $p = 0.032$ ), and four ( $p = 0.008$ ). No differences were observed at hour two ( $p = 0.293$ ) between HH and NH. Oxygen saturation data are presented in **Figure 1**. No differences occurred in heart rate between trials ( $p > 0.05$ ) at baseline, exercise, or immediately post-exercise as no experimental intervention had been introduced at these time-points. Heart rate in the NN condition was lower than HH throughout recovery ( $p < 0.001$ ). Heart rate in the NN condition was lower than NH at hours one ( $p = 0.034$ ), three ( $p < 0.001$ ), and four ( $p < 0.001$ ) with no differences observed at hour two ( $p = 0.066$ ). Furthermore, heart rate was lower in NH compared to HH at recovery hours two ( $p = 0.011$ ), three ( $p = 0.003$ ), and four ( $p = 0.025$ ). Heart rate data are presented in **Figure 2**.

### *Gene Expression*

There were no differences in mitochondria related gene expression of PGC-1 $\alpha$  ( $p = 0.823$ ), ERR $\alpha$  ( $p = 0.970$ ), or GABPA ( $p = 0.913$ ). NRF-1 decreased due to exercise ( $p =$

0.005) but was not different between trials ( $p = 0.750$ ). TFAM increased after both HH ( $p = 0.037$ ) and NH ( $p = 0.006$ ) but not NN ( $p = 0.465$ ) exposure. No differences between HH and NH occurred in TFAM gene expression ( $p = 0.501$ ).

There were no differences in the myogenesis related gene expression of MYOD ( $p = 0.830$ ), MYF-5 ( $p = 0.078$ ) or MYOG ( $p = 0.849$ ) between trials or after exercise ( $p = 0.343$ ,  $p = 0.292$ ,  $p = 0.674$ , respectively). MYF-6 was higher after exercise ( $p = 0.003$ ) but not different between trials ( $p = 0.972$ ). MSTN decreased from pre- to post-exercise ( $p < 0.001$ ) in all conditions and was lower in HH than both NH ( $p = 0.036$ ) and NN ( $p = 0.017$ ). NH and NN conditions were not different from each other ( $p = 0.787$ ). There were no differences in the proteolysis related gene expression of atrogen-1 with exercise ( $p = 0.818$ ) or between trials ( $p = 0.325$ ). However, FOXO3 ( $p = 0.011$ ) and MuRF-1 ( $p < 0.001$ ) gene expression increased with exercise but were not different between conditions ( $p = 0.405$ ,  $p = 0.217$ , respectively). Gene expression data are presented in **Table 3**.

## Discussion

The purpose of the present study was to determine the response of key genes related to mitochondrial development and muscle mass regulation during recovery in HH, NH and NN after exercise. The data from this investigation indicate that arterial oxygen saturation is lower and heart rate is higher in HH recovery compared to NH. Additionally, both hypoxic recovery conditions had lower arterial oxygen saturations and higher heart rates than NN recovery. We also observed increased TFAM gene expression in hypoxic conditions compared to control conditions. Furthermore, we found myostatin, a negative regulator of myogenesis, to be suppressed in HH to a greater extent than NH and NN. While

these differences between HH, NH, and NN were observed, several other genes associated with exercise adaptation were not affected by hypoxia, regardless of the factor used to create the hypoxic environment.

As expected, no differences in arterial oxygen saturation or heart rate occurred at baseline, during exercise, or immediately post-exercise because cycling took place in ambient conditions during each trial. However, recovery in HH produced the lowest arterial oxygen saturation and highest heart rate compared to NH and NN conditions. Our study is in agreement with previous literature, but not all, suggesting cardioventilatory differences exist between hypoxic forms (Loeppky et al., 1997; Savourey, Launay, Besnard, Guinet, & Travers, 2003; A. Tucker, Reeves, Robertshaw, & Grover, 1983). Like the current study, previous data suggest a specific response of HH compared to NH. A lowered barometric pressure modifies fluid circulation and the trans-alveoli-capillary membrane flux (Levine et al., 1988) causing constriction of the pulmonary blood vessels and decrease oxygen diffusion (Millet, Faiss, & Pialoux, 2012) leading to reduced arterial oxygen saturation. Therefore, further increases in heart rate during HH compensate for the reduced oxygen saturation in order to meet metabolic demand.

Recovery in hypoxia increased TFAM gene expression in the current study independent of PGC-1 $\alpha$  or the method in which hypoxia was achieved. TFAM mRNA has previously been shown to increase even in the absence of increased PGC-1 $\alpha$  (Arany et al., 2005; Yin et al., 2008), post-exercise (Pilegaard, Saltin, & Neufer, 2003), or under hypoxic conditions (Gutsaeva et al., 2008; Yin et al., 2008; Zhu et al., 2010). Hypoxia increases TFAM gene expression but does not alter PGC-1 $\alpha$  in mice with ischemic brain injury (Yin et al., 2008). TFAM translocates to the mitochondria and regulates mitochondrial DNA

transcription (Lin, Handschin, & Spiegelman, 2005; Puigserver & Spiegelman, 2003; Wright et al., 2007), which occurs through specific binding to upstream enhancers (Irrcher et al., 2003; Kelly & Scarpulla, 2004; Scarpulla, 2006). These data support the role of TFAM mRNA regulation during hypoxia exposure regardless of whether hypoxia was achieved with an alteration in barometric pressure or inspired oxygen fraction.

Decreases in skeletal muscle mass have been observed after exposure to hypobaric hypoxia during mountaineering expeditions (Hoppeler et al., 1990; Howald & Hoppeler, 2003) where other factors such as exercise and nutrition cannot be controlled. Myostatin is a negative regulator of myogenic signaling - meaning deficiency or inhibition of this gene leads to a muscle growth stimulus (Gumucio & Mendias, 2013; Sandri, 2008). Interestingly, MSTN decreased in all conditions of the current study but was lower in HH than NH and NN. This suggests recovering from exercise in a lower barometric pressure may suppress MSTN to a greater extent than NH or NN recovery. Implications drawn from similar findings demonstrate hypoxic exercise through blood flow occlusion training or whole-body hypoxia exposure increases muscle size and strength (Manimmanakorn et al., 2013; Nishimura et al., 2010). Specifically, resistance training with blood flow occlusion significantly reduces MSTN mRNA expression compared to resistance training alone (Laurentino et al., 2012). Despite potential differences in hypoxic mechanisms, it appears as if blood flow occlusion causes a hypoxic state similar to HH in terms of MSTN inhibition. Myostatin attenuation in these environments may provide a myogenic stimulus, particularly after exercise. Therefore, if HH recovery from exercise further attenuates MSTN, protocols may be developed to suppress MSTN expression further and potentially lead to a greater muscle-building stimulus. Applied research investigating the changes in

muscle mass and strength incorporating hypobaria into a training regimen is needed in order to further develop this hypothesis.

Despite the differences that occurred between HH, NH, and NN, we observed no other effects of hypoxic recovery after exercise on select skeletal muscle gene expression. We observed a lower than expected exercise stimulated response in the genes we measured. However the exercise response in human gene expression appears to be affected by several variables and is generally in agreement with previous literature on mitochondrial biogenesis (Cartoni et al., 2005; Hock & Kralli, 2009; McGee & Hargreaves, 2004; Pilegaard et al., 2003; Tunstall et al., 2002), myogenesis (Coffey et al., 2006; Yang, Creer, Jemiolo, & Trappe, 2005), and proteolysis (Harber et al., 2009; Raue, Slivka, Jemiolo, Hollon, & Trappe, 2007; Sandri et al., 1995). Indeed, a subject population or exercise protocol that yielded a more robust response in gene expression may lead to a differential effect of hypoxia.

## **Conclusion**

When recovery from exercise takes place in hypobaric hypoxia (HH), lower oxygen saturations (SpO<sub>2</sub>) and higher heart rates occur compared to normobaric hypoxia (NH) recovery. Additionally, HH recovery attenuates myostatin (MSTN) to a greater extent than NH and NN after exercise. These data suggest a lowered barometric pressure initiates a greater hypoxic response compared to a lowered fractional oxygen concentration on these measures. However, hypoxia did not affect several other genes associated with exercise adaptation, regardless of the factor used to create the hypoxic environment.

## **Disclosure Statement**

No competing financial interests exist.

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## Tables

**Table 1:** Probes and Primers used for real-time reverse transcription quantitative PCR

Reference Genes	Primer 1	Primer 2	Probe
ACTB	AAGTCAGTGTACAGGTAAGCC	GTCCCCCAACTTGAGATGTATG	CTGCCTCCACCCACTCCC A
B2M	ACCTCCATGATGCTGCTTAC	GGACTGGTCTTTCTATCTCTTGT	CCTGCCGTGTGAACCATGTGACT
CYC	TCTTTCACCTTTGCCAAACACC	CATCCTAAAGCATACGGGTCC	TGCTTGCCATCCAACCACTCAGTC
RPS18	GTCAATGTCTGCTTTCTCTAAC	GTTCCAGCATATTTTTCGAGT	TCTTCGGCCCCACACCCTTAATGG
GAPDH	TGTAGTTGAGGTCAATGAAGGG	ACATCGCTCAGACACCATG	AAGGTCGGAGTCAACGGATTTGGTC
Mitochondria Genes	Primer 1	Primer 2	Probe
PGC-1 $\alpha$	TGTCTGTATCCAAGTCGTTTAC	GAGTCTGTATGGAGTGACATCG	ACCAGCCTCTTTGCCAGATC TTC
ERR $\alpha$	TCTCCGCTTGGTGATCTCA	CTATGGTGTGGCATCCTGTG	TGGTCCTCTTGAAGAAGGCTTTGCA
GABPA	TGTAGTCTTGGTTCTAGCAGTTTC	TGGAACAGAGAAAGCAGAGTG	TGGTTCATTGATGTCTATGGCCTGGC
NRF-1	GTCATCTCACCTCCCTGTAAC	GATGCTTCAGAATTGCCAACC	ATGGAGAGGAACAAAATTGGGC
TFAM	GCCAAGACAGATGAAAACCAC	TGGGAAGGTCTGGAGCA	CGCTCCCCCTTCAGTTTTGTGTATTT
Myogenic Genes	Primer 1	Primer 2	Probe
MYOD	GAGATGCGCTCCACGATG	CGGAAGTGTACGAAGGC	ACAGGCAGTCTAGGCTCGACAC
MYOG	AGAAGTAGTGGCATCTGTGG	GACAGCATCACAGTGGAAGA	ATGCCC GGCTTGGAAGACAATCT
MSTN	TCGTGATTCTGTTGAGTGCT	TGTAACCTTCCCAGGACCA	TCTTTTTGGTGTGTCTGTTACCTTGACCT
MYF-5	GGCATATACATTTGATACATCAGGAC	CACCTCCAAGTGTCTGTATG	TGCTGTCAAAAGTACTGCTCTTTCTGGA
MYF-6	CTACTCGAGGCTGACGAATC	CAGCTACAGACCCAAACAAGA	TGATAACGGCTAAGGAAGGAGGAGCA
Proteolytic Genes	Primer 1	Primer 2	Probe
FOXO3	CGTGCCCTACTTCAAGGATAAG	ATTCTGGACCCGCATGAATC	AGGTTGTGCCGGATGGAGTTCTTC
Atrogin-1	TCAGCCTCTGCATGATGTTT	CAACAGACTGGACTTCTCAACT	CACTGACCTGCCTTTGTGCCTACA
MURF-1	GCAACTCACTTTTCTTCTCATCC	TGCAGACCATCATCACTCAG	ACCTGGTGACTGTTCTCCTTGGTC

**Table 2.** Participant Descriptive Data

Measures	Males (n = 8)	Females (n = 7)	Combined (n = 15)
Age (y)	24 ± 0.93	24 ± 1.81	24 ± 1.04
Height (cm)	184 ± 1.63†	166 ± 3.15	178 ± 3.14
Weight (kg)	78.97 ± 2.71*	65.23 ± 4.13	72.47 ± 3.57
Body Fat (%)	12 ± 0.01*	24 ± 0.03	14 ± 0.02
VO <sub>2</sub> peak (L · min <sup>-1</sup> )	4.24 ± 0.15*	3.04 ± 0.15	3.60 ± 0.20
Watt max (W)	335 ± 8.56†	219 ± 14.92	281 ± 17.88

Data are means ± SE. \* p < 0.05 males different from females, † p < 0.001 males different from females.

**Table 3.** Fold change in genes related to mitochondrial development, myogenesis, and proteolysis after exercise and during recovery in Normobaric Normoxic (NN), Normobaric Hypoxic (NH), and Hypobaric Hypoxic (HH) conditions.

Genes	NN		NH		HH		Grand means	
Mitochondrial	Pre	4h Post	Pre	4h Post	Pre	4h Post	Pre	4h Post
PGC-1α	1.006 ± 0.001	1.133 ± 0.297	1.011 ± 0.005	1.283 ± 0.296	1.019 ± 0.012	0.888 ± 1.006	1.012 ± 0.006	1.089 ± 0.241
ERRα	1.047 ± 0.016	1.133 ± 0.297	1.014 ± 0.005	1.146 ± 0.209	1.049 ± 0.017	1.090 ± 0.209	1.037 ± 0.013	1.123 ± 0.238
GABPA	1.017 ± 0.007	0.877 ± 0.100	1.014 ± 0.005	1.129 ± 0.228	1.015 ± 0.008	1.124 ± 0.232	1.015 ± 0.007	1.044 ± 0.187
NRF-1	1.011 ± 0.005	0.819 ± 0.116	1.010 ± 0.003	0.942 ± 0.102	1.023 ± 0.012	1.008 ± 0.810	1.015 ± 0.007	0.923 ± 0.142*
TFAM	1.008 ± 0.002	0.895 ± 0.079	1.021 ± 0.008	1.508 ± 0.177‡	1.012 ± 0.003	1.311 ± 0.155‡	1.014 ± 0.004	1.238 ± 0.137
Myogenesis	Pre	4h Post	Pre	4h Post	Pre	4h Post	Pre	4h Post
MYOD	1.047 ± 0.014	2.430 ± 0.825	1.058 ± 0.018	1.631 ± 0.416	1.065 ± 0.014	1.593 ± 0.334	1.057 ± 0.015	1.885 ± 0.525
MSTN	1.029 ± 0.011	0.495 ± 0.093	1.017 ± 0.006	0.489 ± 0.088†	1.008 ± 0.002	0.284 ± 0.051‡	1.018 ± 0.006	0.423 ± 0.077*
MYOG	1.005 ± 0.002	1.139 ± 0.206	1.015 ± 0.008	1.026 ± 0.104	1.017 ± 0.011	1.148 ± 0.140	1.012 ± 0.007	1.104 ± 0.150
MYF-5	1.023 ± 0.011	1.025 ± 0.275	1.021 ± 0.008	1.862 ± 0.618	1.016 ± 0.007	1.174 ± 0.258	1.02 ± 0.008	1.354 ± 0.384
MYF-6	1.013 ± 0.006	1.671 ± 0.209	1.021 ± 0.010	1.734 ± 0.381	1.012 ± 0.005	1.901 ± 0.446	1.015 ± 0.007	1.768 ± 0.346*
Proteolysis	Pre	4h Post	Pre	4h Post	Pre	4h Post	Pre	4h Post
FOXO3	1.016 ± 0.006	1.892 ± 0.372	1.022 ± 0.009	1.338 ± 0.159	1.019 ± 0.009	2.567 ± 0.802	1.019 ± 0.008	1.932 ± 0.445*
Atrogin-1	1.105 ± 0.078	2.047 ± 0.522	1.025 ± 0.009	0.920 ± 0.141	1.079 ± 0.053	2.441 ± 1.012	1.07 ± 0.047	1.802 ± 0.559
MURF-1	1.022 ± 0.007	4.245 ± 0.828	1.013 ± 0.005	2.627 ± 0.529	1.006 ± 0.001	3.775 ± 0.669	1.013 ± 0.004	3.549 ± 0.676*

\* p < 0.05 from pre, † p < 0.05 from post Hypobaric Hypoxia, ‡ p < 0.05 from post Normobaric Normoxia. Data are ± SE.

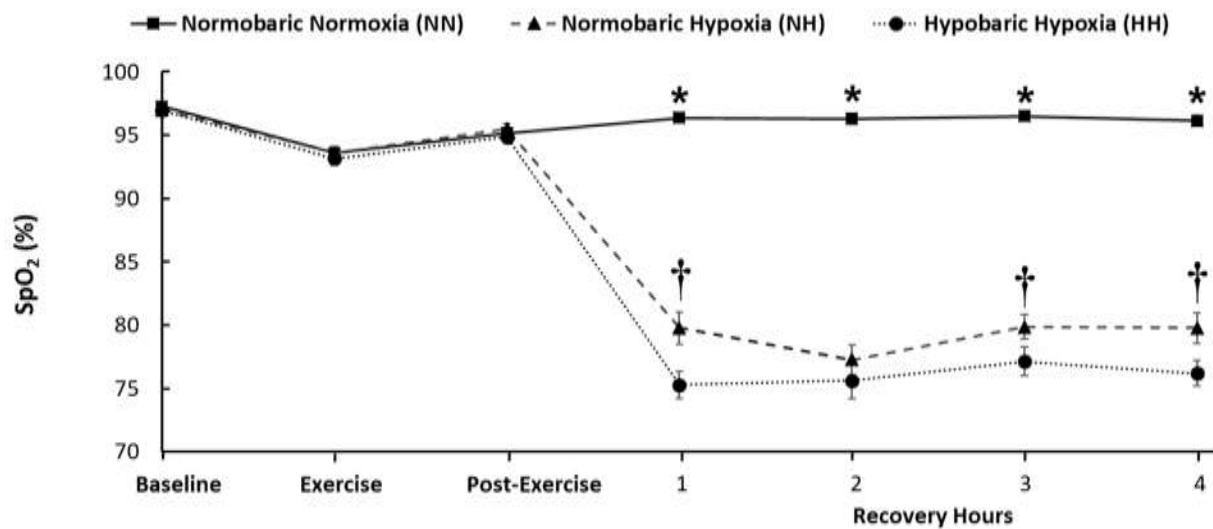


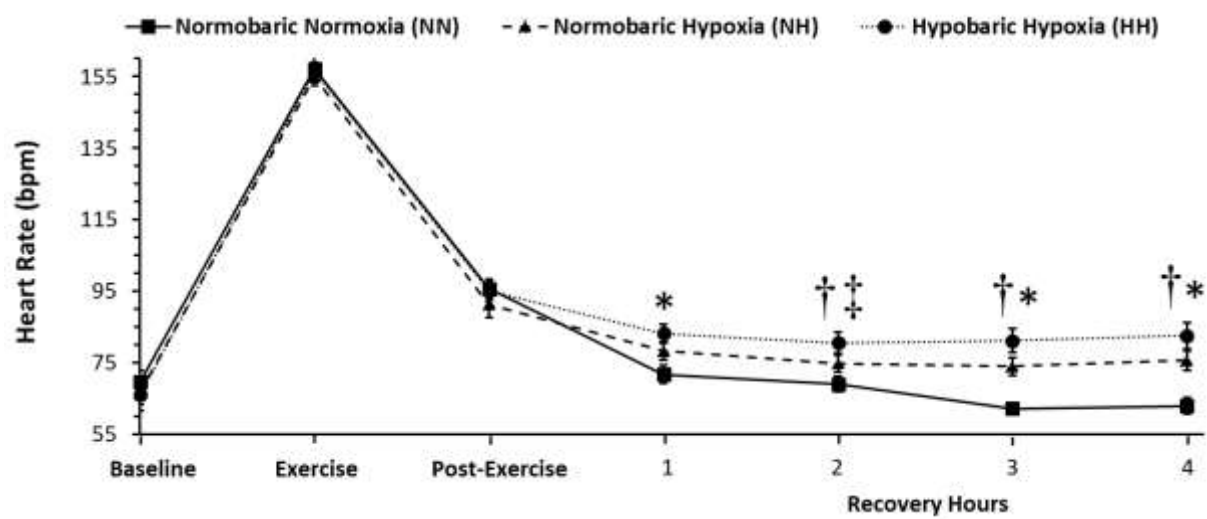
## Figure Legends

**Figure 1.** Oxygen saturation between trials at baseline, during exercise, immediately post-exercise and each hour of recovery. \*  $p < 0.05$  NN from NH and HH, †  $p < 0.05$  NH from HH.

**Figure 2.** Heart rate between trials at baseline, during exercise, immediately post-exercise and each hour of recovery. \*  $p < 0.05$  NN from NH and HH, †  $p < 0.05$  NH from HH ‡  $p < 0.05$  NN from HH.

## Figures





## **7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS**

### **Montana**

Name: Brent Ruby

Project Role: PI

Researcher Identifier (e.g. ORCID ID): NA

Nearest person month worked: 6

Contribution to Project: Dr. Ruby coordinated study design, implementation, sample and data collection, and reporting.

Funding Support:

Name: Walter Hailes

Project Role: Research Associate

Researcher Identifier (e.g. ORCID ID): NA

Nearest person month worked: 6

Contribution to Project: Mr. Hailes coordinated study participant recruitment and management and organized/conducted data collection.

Funding Support:

Name: John Cuddy

Project Role: Research Associate

Researcher Identifier (e.g. ORCID ID): NA

Nearest person month worked: 6

Contribution to Project: Mr. Cuddy contributed to study participant recruitment and management and organized/conducted data collection.

Funding Support:

Name: John Quindry

Project Role: Co-investigator

Researcher Identifier (e.g. ORCID ID): NA

Nearest person month worked: 2

Contribution to Project: Dr. Quindry is organizing data analyses for the oxidative stress markers.

Funding Support:

### **Nebraska**

Name: Dustin Slivka

Project Role: Co-PI

Researcher Identifier (e.g. ORCID ID): NA

Nearest person month worked: 6

Contribution to Project: Dr. Slivka assisted in study design, implementation, sample analysis, statistical analysis, and reporting.

Funding Support:

Name: Roksana Zak  
Project Role: Graduate Student (doctoral)  
Researcher Identifier (e.g. ORCID ID): NA  
Nearest person month worked: 6  
Contribution to Project: Ms. Zak performed skeletal muscle gene expression analysis  
Funding Support:

Name: Caleb Ross  
Project Role: Graduate Student (masters)  
Researcher Identifier (e.g. ORCID ID): NA  
Nearest person month worked: 6  
Contribution to Project: Mr. Ross assisted in the muscle processing and analysis.  
Funding Support:

## **8. SPECIAL REPORTING REQUIREMENTS**

**Quad Chart:** See attached.